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Purification, Characterization, and Genetic Analysis of *Mycobacterium tuberculosis* Urease, a Potentially Critical Determinant of Host-Pathogen Interaction

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Mycobacterium tuberculosis **urease (urea amidohydrolase [EC 3.5.1.5]) was purified and shown to contain three subunits: two small subunits, each approximately 11,000 Da, and a large subunit of 62,000 Da. The N-terminal sequences of the three subunits were homologous to those of the A, B, and C subunits, respectively, of other bacterial ureases. *M. tuberculosis* urease was specific for urea, with a K_m of 0.3 mM, and did not hydrolyze thiourea, hydroxyurea, arginine, or asparagine. The enzyme was active over a broad pH range (optimal activity at pH 7.2) and was remarkably stable against heating to 60°C and resistant to denaturation with urea. The enzyme was not inhibited by 1 mM EDTA but was inhibited by *N*-ethylmaleimide, hydroxyurea, acetohydroxamate, and phenylphosphorodiamidate. Urease activity was readily detectable in *M. tuberculosis* growing in nitrogen-rich broth, but expression increased 10-fold upon nitrogen deprivation, which is consistent with a role for the enzyme in nitrogen acquisition by the bacterium. The gene cluster encoding urease was shown to have organizational similarities to urease gene clusters of other bacteria. The nucleotide sequence of the *M. tuberculosis* urease gene cluster revealed open reading frames corresponding to the urease A, B, and C subunits, as well as to the urease accessory molecules F and G.**

Tuberculosis is a global health problem of escalating proportions. *Mycobacterium tuberculosis*, the main etiologic agent of tuberculosis, is a facultative intracellular bacterial pathogen that parasitizes host mononuclear phagocytes. Throughout its life cycle within the mononuclear phagocyte, *M. tuberculosis* resides in a membrane-bound phagosome which is only mildly acidified (5) and which does not fuse with lysosomes (1, 4, 41). However, the mechanisms underlying these phenomena are unknown.

Previous studies have demonstrated that NH_4Cl added exogenously to mouse mononuclear phagocytes blocks phagosome-lysosome fusion (6) and promotes phagosome endosome fusion (8). Hence, ammonia production by intracellular *M. tuberculosis* may partly underlie the inhibition of phagosome-lysosome fusion. *M. tuberculosis* is able to produce ammonia from urea by the action of urease, and thus this enzyme may play a role in phagosome-lysosome fusion inhibition. In addition, urease may modulate the pH of the phagosome and provide a source of nitrogen for biosynthesis. However, urease of *M. tuberculosis* has not been purified or characterized previously. To learn more about *M. tuberculosis* urease, we have purified and characterized the enzyme and cloned and sequenced the urease gene cluster.

MATERIALS AND METHODS

Biochemicals and chemicals. *Bacillus pasteurii* urease, jack bean urease, urea, α-ketoglutarate, NADPH, glutamate dehydrogenase, thiourea, hydryoxylurea, *N*-ethylmaleimide, and acetohydroxamate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phenylphosphorodiamidate was obtained from Lancaster Chemical Co. (Windham, N.H.). Q-Sepharose fast-flow, Superdex-75, and phenyl-Sepharose were purchased from Pharmacia (Piscataway, N.J.).

Bacterial strains. Virulent *M. tuberculosis*, Erdman strain, was obtained from the American Type Culture Collection (ATCC 35801) and maintained in 7H9 Middlebrook medium (Difco) at pH 6.7, 37°C, and 5% CO_2. Cultures were grown for 3 weeks from an initial optical density at 540 nm of 0.05, corresponding to 3×10^7 cells per ml, to a final optical density of 0.3 to 0.5, corresponding to approximately 5×10^8 cells per ml. *Escherichia coli* DH5α F^- ϕ80d*lacZ*Δ (*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_K^- m_K^+$) *supE44*λ$^-$ *thi-1 gyrA96 relA1* (Gibco BRL, Gaithersburg, Md.) was used for cloning purposes.

Purification of urease. Bacterial cell pellets were obtained by filtration on 0.8-μm-pore-size filters, resuspended in hypotonic medium (20 mM Bis-Tris, pH 7.0, containing 1 mM EDTA), and sonicated with a probe tip sonicator (model W-375; Heat Systems Ultrasonics, Plainview, N.Y.) at 50% of the work cycle at maximum sonicator output for a total of 30 min. The sample was stirred continuously with a magnetic stirring bar and chilled in an ice bath during sonication. The sample was sonicated inside a sealed container placed within a biohazard hood.

The sonicate was centrifuged at 16,200 × *g* for 1 h at 4°C, and the supernatant was passed sequentially through 0.4- and 0.2-μm-pore-size filters. The filtrate was loaded onto a Q-Sepharose anion-exchange column and was eluted first with a 0 to 0.3 M NaCl gradient in 20 mM Bis-Tris, pH 7.0, containing 1 mM EDTA and then with a 0.3 to 0.5 M NaCl gradient. Fractions with urease activity were pooled and concentrated with a Centriprep 30 concentrator (Amicon, Beverly, Mass.), applied to a Superdex-75 gel filtration column, and eluted with 20 mM bis-Tris HCl, pH 7.0, containing 150 mM NaCl and 1 mM EDTA. Fractions with urease activity were pooled, and solid NaCl was added slowly with continuous stirring to achieve 1 M NaCl. The sample was then loaded onto a phenyl-Sepharose column and eluted with a 1 to 0 M NaCl gradient. Fractions with urease activity were again concentrated with a Centriprep 30 concentrator.

Assay for urease activity and protein concentration. Urease activity was assayed by incubating 5 to 10 μl of enzyme sample in 0.5 ml of 0.1 M urea in 0.1 M bis-Tris, pH 7.2, at 37°C. After 30 to 90 min, 10-μl aliquots were removed from the reaction mixture for measurement of the ammonia concentration. Ammonia was quantitated by measuring the decrease in the A_{340} in an assay involving the conversion of NADPH to NADP in the presence of glutamate dehydrogenase (40). This measurement was made by using a 200-μl reaction volume (1.2 U of glutamate dehydrogenase per ml, 0.23 mM NADPH, 3.4 mM α-ketoglutarate) in 96-well multititer plates (Costar, Cambridge, Mass.) and a Titertek MCC 340 microtiter plate reader (Flow Laboratories, McLean, Va.). The generation of ammonia by urease was linear with enzyme concentration and with time from 30 to 90 min.

The protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.).

Examination of urease expression in response to nitrogen starvation and other environmental conditions. *M. tuberculosis* was subcultured in Sauton's medium or modified Sauton's medium containing various amounts of asparagine for 1 week at 37°C in a 5% CO_2 atmosphere. Bacterial pellets were collected by centrifugation at 10,000 × *g* for 10 min at 4°C in aerosol-tight biohazard capsules (Brinkmann Instruments, Westbury, N.Y.). The pellets were then sonicated with

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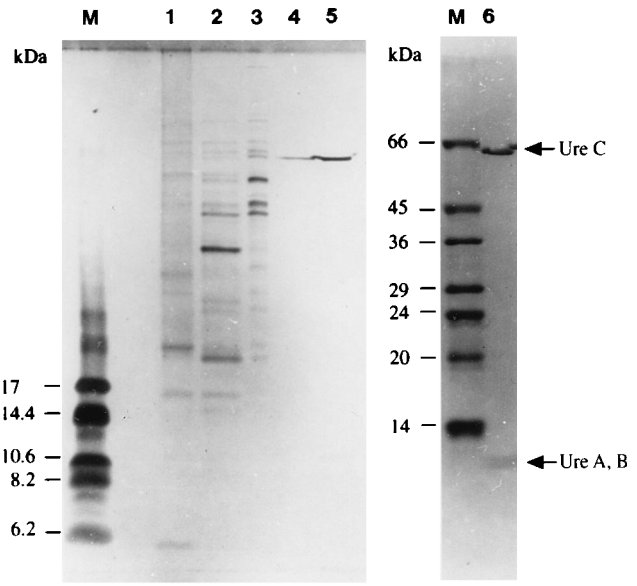

FIG. 1. Tricine-sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis of *M. tuberculosis* urease after successive steps of purification. Lanes: 1, crude extract, 1.0 μg of protein; 2, Q-Sepharose, 1.0 μg of protein; 3, gel filtration, 1.0 μg of protein; 4, phenyl-Sepharose, 0.2 μg of protein; 5, phenyl-Sepharose, 1.0 μg of protein; 6, phenyl-Sepharose, 10 μg of protein; M, standard molecular mass markers (sizes are indicated). Lanes 1 to 5 are stained with silver nitrate. Lane 6 is stained with Coomassie blue. The urease A and B subunits were stained poorly with silver nitrate but were stained more strongly with Coomassie blue.

a microprobe tip sonicator (Heat Systems Ultrasonics) for 30 min at 0°C and centrifuged at 30,000 × *g* for 30 min, and the supernatants were passed through 0.2-μm-pore-size filters. The filtered supernatants were assayed for urease activity and protein concentration.

Native and denaturing gel electrophoresis. Native polyacrylamide gel electrophoresis and detection of urease on the native gel was performed as described by Mobley et al. (25). Denaturing gel electrophoresis was performed with 15% polyacrylamide gels as described by Laemmli (14) and 10% Tricine gels as described by Schagger and von Jagow (33).

N-terminal sequencing. N-terminal sequences of peptides were determined by blotting partially purified proteins onto polyvinylidene difluoride membranes, staining the membrane with Coomassie brilliant blue, destaining with 50% methanol, and subjecting the band to automated Edman degradation. N-terminal sequencing was performed by the University of California, Los Angeles, protein microsequencing core facility.

Nickel content. Nickel content was determined by atomic absorption spectrometry, kindly performed by Thomas Moyer, Mayo Clinic, Rochester, Minn.

DNA manipulations. Recombinant-DNA techniques were performed as described previously (22). DNA fragments used as probes in Southern blot hybridizations and colony hybridizations were radiolabelled by random priming with [α-^{32}P]dCTP by the multiprime labelling system (kit RPN.160 1Y; Amersham, Arlington Heights, Ill.). Oligonucleotides used as sequencing primers or probes were synthesized by Biosynthesis Corporation (Lewisville, Tex.) and 5′ end labelled with [γ-^{32}P]ATP (Amersham) when used as probes. Southern blot DNA hybridization and colony hybridizations with oligonucleotide probes (10^6 cpm/ml) were performed with nitrocellulose filters for 16 h at 45°C in Denhardt's solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 μg of sonicated salmon sperm DNA per ml, 50 mM sodium phosphate buffer (pH 6.8), 1 mM sodium PP_i, 100 μM ATP, and 20% formamide (39).

DNA sequencing and sequence analysis. DNA sequencing was performed by using α-^{35}S-dCTP (Amersham) and the Takara Ladderman sequencing kit (Panvera, Madison, Wis.) according to the manufacturer's directions. Sequencing gels contained 6% polyacrylamide and 50% urea (National Diagnostics, Atlanta, Ga.). Sequences were confirmed by sequencing both strands in opposite directions. DNA sequences were submitted to the National Center for Biotechnology Information for homology searches and comparisons with other sequences in GenBank. The molecular weights and pIs of deduced amino acid sequences were determined by using MacProMass computer software (18).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession no. MTB U33011.

RESULTS

Purification of *M. tuberculosis* urease. We purified urease from the bacterial pellets of 3-week-old *M. tuberculosis* Erdman cultures. *M. tuberculosis* urease was found to be present primarily in the cell pellet, with less than 5% of urease activity in the culture filtrate. The purification procedure gave a 1,000-fold enrichment of the enzyme activity and an overall yield of purified enzyme of 2% (Table 1). The enzyme consisted of three subunits as determined by denaturing polyacrylamide gel electrophoresis: a prominent band of approximately 62,000 Da and a weakly staining doublet of approximately 11,000 Da (Fig. 1). The doublet at 11,000 Da was stained weakly by silver nitrate but somewhat more strongly by Coomassie blue (Fig. 1). Microsequencing of these bands revealed three N-terminal peptide sequences with high degrees of similarity to the urease A, B, and C subunits of other bacterial ureases. The first five residues of the A and B subunits (MRLTP and MIPGE, respectively) and the first 27 residues of the C subunit (ARLSR ERYAQLYGPTTGDRIRLADTNL), determined by peptide microsequencing, were 100% identical with sequences predicted from the corresponding open reading frames of the urease A, B, and C genes, which were determined by cloning and sequencing the urease gene cluster of *M. tuberculosis* Erdman genomic DNA (see below).

M. tuberculosis urease activity was always eluted as a single symmetrical peak by gel filtration and by phenyl-Sepharose chromatography. However, two peaks were typically observed during Q-Sepharose ion-exchange chromatography: a major peak of enzyme activity which was eluted at 350 mM NaCl and a much smaller peak of enzyme activity which was eluted at 370 mM NaCl. Occasionally a small peak of enzyme activity was also eluted at 5 mM NaCl. It is possible that this microheterogeneity is attributable to limited proteolysis of the macromolecule resulting in a significant change in charge but not mass or hydrophobicity. On nondenaturing zymogram gels, *M. tuberculosis* urease enzyme activity appeared as a doublet, with one prominent band and a faster-migrating relatively minor band (Fig. 2). Multiple isoenzyme forms have been observed for several other bacterial ureases, but the basis for this multiplicity is not understood (26).

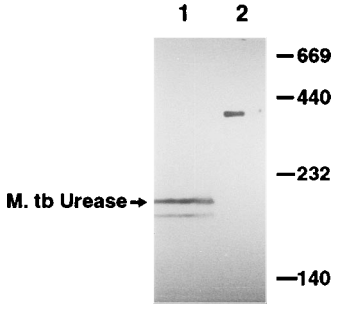

FIG. 2. Nondenaturing gel electrophoresis of *M. tuberculosis* (M. tb) urease activity. *M. tuberculosis* cell pellets were lysed by sonication in hypotonic medium, fractionated by Q-Sepharose anion-exchange chromatography, and electrophoresed on a 6% polyacrylamide gel with a 4% stacking gel. The gel was equilibrated with 0.02% neutral red–0.1% EDTA and incubated with 1.5% urea. Lane 1, *M. tuberculosis* urease. Lane 2, jack bean urease. The arrow indicates the major band of *M. tuberculosis* urease activity; a fainter band of urease activity is present just below the major band. On the original gel, the bands appeared red against a yellow background. For comparison, the migration positions of standard proteins with known molecular masses are shown (in kilodaltons) (thyroglobulin, 669,000 Da; apoferritin, 440,000 Da; catalase, 232,000 Da; and lactate dehydrogenase, 140,000 Da).

TABLE 1. Purification of *M. tuberculosis* urease

Step	Amt of protein	Total urease activity (μmol of NH_3/min)	Yield (%)	Urease sp act (μmol of NH_3/min/mg of protein)	Fold purification
Crude sonicate	871 mg	82.6	100	0.1	1
Q-Sepharose	5.9 mg	30.3	36.7	5.2	54
Gel filtration	1.4 mg	9.4	11.4	6.6	69
Phenyl-Sepharose	18 μg	1.8	2.1	101	1,063

Molecular weight and subunit composition of *M. tuberculosis* urease. We measured the molecular weight of native urease by gel filtration on both Sephacryl S-300 HR and Superdex-75 columns (data not shown). Both columns yielded an apparent native M_r of 185,000. This is similar to the apparent M_rs of ureases of several other microorganisms, such as *Proteus mirabilis* (3) and *B. pasteurii* (15).

The ureases of several bacteria (*P. mirabilis*, *Morganella morganii*, and *Providencia stuartii*) have been reported to be heteropolymers with three distinct polypeptide chains with a proposed $A_4B_4C_2$ stoichiometry (26). The proposed stoichiometry has been based upon the apparent M_r as determined by gel filtration and the relative intensity of the staining of the subunits on denaturing polyacrylamide gels. However, Jabri and coworkers (10) have recently reported a high-resolution X-ray crystallographic study of *Klebsiella aerogenes* urease and demonstrated that this urease has a trimeric structure with an $A_3B_3C_3$ stoichiometry. In view of the homology between the ureases of *M. tuberculosis* and *K. aerogenes*, it is likely that *M. tuberculosis* urease also has a stoichiometry of $A_3B_3C_3$. Assuming that *M. tuberculosis* urease has an $A_3B_3C_3$ stoichiometry, its predicted native M_r is approximately 250,000, which is substantially higher than the apparent M_r determined by gel filtration.

Nickel content of *M. tuberculosis* urease. Measurement of the Ni^{2+} content by atomic mass absorption spectrometry revealed 1.4 ng of Ni^{2+} per μg of purified urease. Assuming a mass of 250 kDa, this is consistent with a total of six Ni^{2+} ions per urease molecule (or two nickel ions per active site on each of the C catalytic subunits). The X-ray crystallography study of *K. aerogenes* urease (10) and earlier atomic absorption spectrometry studies of *P. stuartii* (28) and *K. aerogenes* (36) ureases also suggested the presence of two nickel ions per catalytic subunit.

Enzymology of *M. tuberculosis* urease. (i) Kinetics. We measured the rate of ammonia generation by purified urease at different concentrations of urea by using a glutamate dehydrogenase–NADPH-coupled assay system (data not shown). *M. tuberculosis* urease had a K_m for urea of 0.3 mM, a value which is lower than the value reported for most other ureases (2 to 15 mM) (26). In our study, the K_m of jack bean urease was 5 mM, which is in good agreement with the values determined by others. The low K_m of *M. tuberculosis* urease indicates that this urease can generate ammonia at lower substrate concentrations than many other ureases. It is likely that the concentration of urea available to the enzyme inside the *M. tuberculosis* phagosome is lower than that available to bacteria whose niche is the urinary tract. Interestingly, urease of *Helicobacter pylori* also exhibits a K_m of 0.3 mM, and, like *M. tuberculosis* urease, this urease is also probably exposed to relatively low plasma urea levels. The maximal reaction velocity (V_{max}) of *M. tuberculosis* urease was 101 μmol of NH_3 per min per mg of protein, which is lower than the velocity reported for other ureases (1,000 to 5,000 μmol/min/mg). Under our assay conditions, jack bean urease had a V_{max} of 1,200 μmol/min/mg. It should be noted that *M. tuberculosis* is the first intracellular pathogen for which urease has been studied, and its properties may be unique in several respects.

(ii) Substrate specificity. *M. tuberculosis* urease was specific for urea and did not hydrolyze arginine, asparagine, hydroxyurea, or thiourea. Crude sonicates of *M. tuberculosis* cell pellets did show asparaginase and arginase activities (measuring 0.3

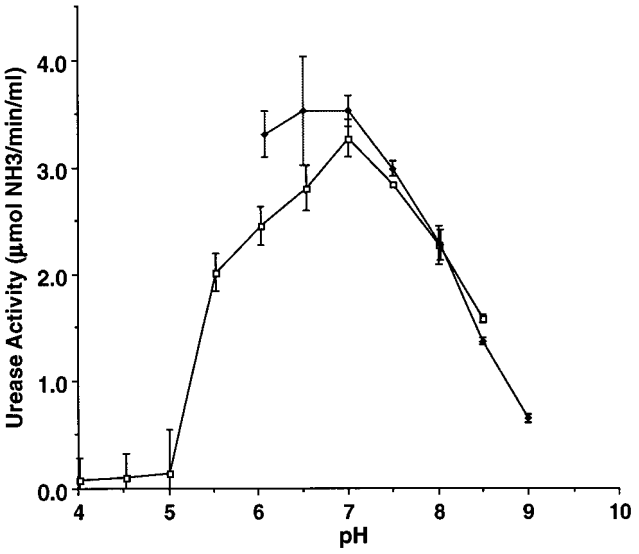

FIG. 3. Effect of pH on urease activity of *M. tuberculosis*. Purified urease of *M. tuberculosis* was incubated with 0.1 M urea in either 0.1 M morpholineethanesulfonic acid (□) or 0.1 M *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (◆) at the indicated pH. Ammonia released by the hydrolysis of urea was detected by a glutamate dehydrogenase–NADPH-coupled reaction. *M. tuberculosis* urease shows activity over a broad pH range, from 5.5 to 8, with optimal activity at pH 7.2. Data shown are means ± SD of triplicate determinations.

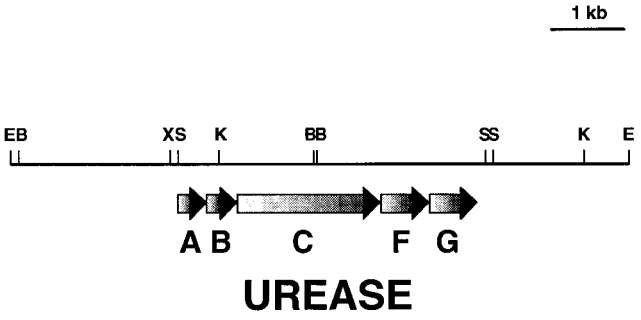

FIG. 4. Organization of the *M. tuberculosis* urease gene cluster. *E. coli* clones containing the *M. tuberculosis* urease gene cluster were identified by screening *M. tuberculosis* genomic libraries with a probe derived from the N-terminal protein sequence of the urease C subunit. Nucleotide sequencing of the urease gene cluster revealed three open reading frames for the urease A, B, and C subunits and two open reading frames for the urease accessory molecules F and G. Restriction endonuclease sites are indicated as follows: E, *Eco*RI; B, *Bam*HI; X, *Xho*I; S, *Sph*I; and K, *Kpn*I.

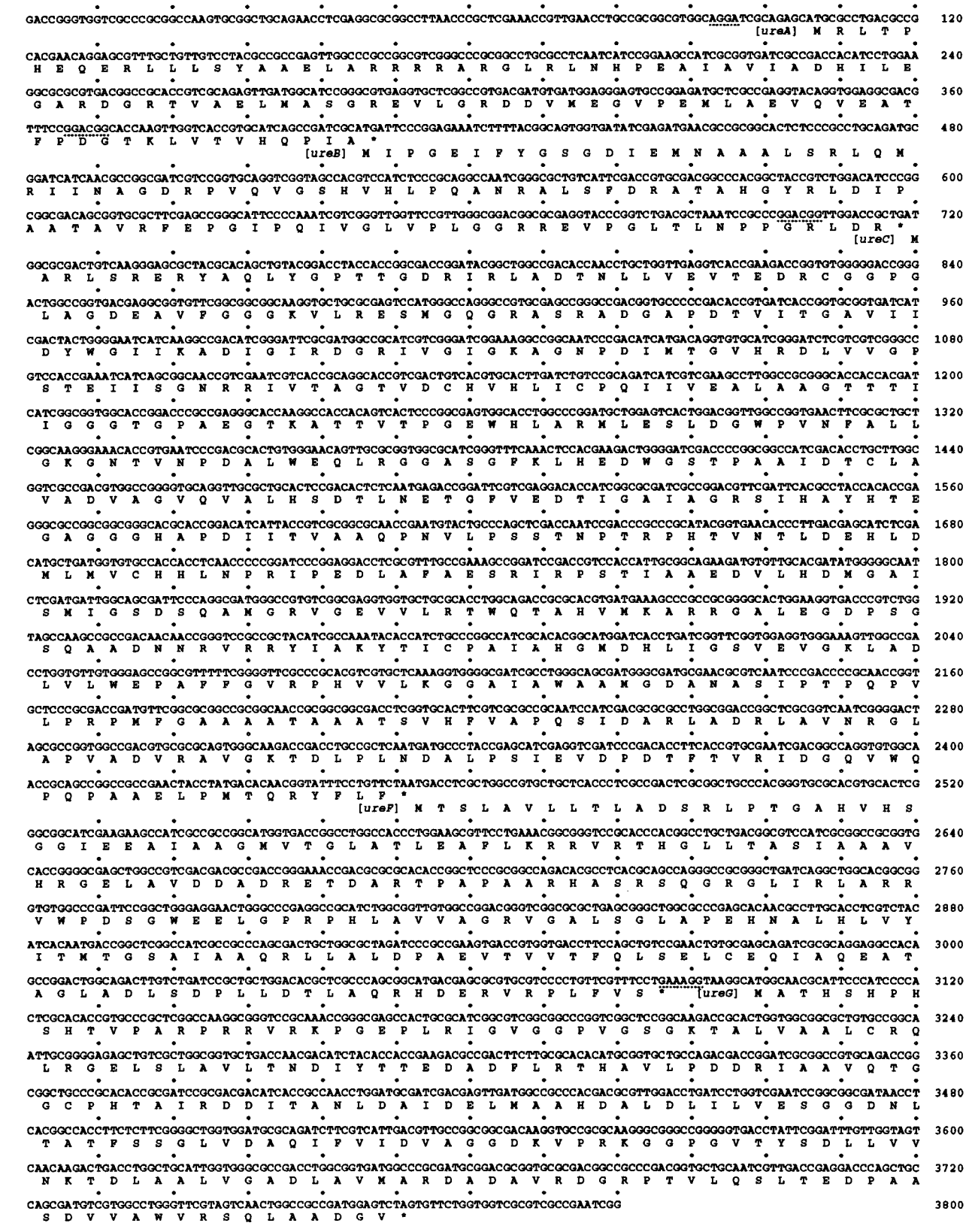

FIG. 5. Nucleotide sequence of the *M. tuberculosis* urease gene cluster and predicted amino acid sequences of the *M. tuberculosis* urease A, B, and C subunits and urease accessory molecules F and G. Shine-Dalgarno consensus sequences upstream of *ureA*, *ureB*, *ureC*, and *ureG* are underlined with dashed lines. Stop codons are indicated by asterisks.

TABLE 2. Effect of nitrogen starvation on urease expression by *M. tuberculosis*

[Asn] (mM)	Protein concn (mg/ml)	Urease activity (nmol of NH_3/ml/min)	Urease sp act (nmol of NH_3/min/mg of protein)	Fold increase in urease sp act
15	0.47 ± 0.01	25 ± 2	53	1
1.5	0.17 ± 0.02	39 ± 8	230	4.3
0.75	0.08 ± 0.00	31 ± 3	396	7.4
0.375	0.04 ± 0.00	20 ± 1	499	9.4

and 1% of the urease activity, respectively, expressed as micromoles of NH_3 per minute per microgram of protein). Both asparaginase and arginase activities separated from urease activity during anion-exchange chromatography on Q-Sepharose (data not shown).

(iii) pH-activity relationship. *M. tuberculosis* urease shows enzyme activity over a broad pH range (Fig. 3), between 5.5 and 8, with maximal activity at pH 7.2. Maximal enzyme activity in the pH range of 5.5 to 7.5 is ideal for a pathogen such as *M. tuberculosis* residing in a mildly acidified phagosome (5). Other bacterial ureases tend to have a slightly more alkaline pH profile, with maximal activity at pH 8. Ureases of urinary pathogens are exposed to higher-pH environments because of alkalinization of the infected urine.

(iv) Inhibitors of *M. tuberculosis* urease activity. Several different classes of inhibitors of jack bean and microbial ureases have been described. First, compounds such as thiourea (31) and hydroxyurea (25) act as substrate analog inhibitors. Second, sulfhydryl-reactive compounds such as *N*-ethylmaleimide inhibit enzyme activity by reacting with a free cysteine sulfhydryl group required for enzyme activity (38). Third, acetohydroxamate (21, 29) is a slowly binding competitive inhibitor thought to inhibit urease by chelating Ni^{2+} at the active site of the enzyme (37). Fourth, phosphoramide derivatives, such as phenylphosphorodiamidate, are the most potent inhibitors of ureases, with K_i values of less than 0.1 nM (12, 23, 37). We have compared the inhibition profiles for each of these inhibitors for ureases from jack bean, *B. pasteurii*, and *M. tuberculosis*. Hydroxyurea, *N*-ethylmaleimide, and acetohydroxamate inhibited the urease of *M. tuberculosis* (50% inhibitory concentrations of 1 mM, 0.1 mM, and 2 μM, respectively) to an extent similar to their effects on the ureases of jack bean and *B. pasteurii*. Preincubation with acetohydroxamate was necessary to achieve complete inhibition of *M. tuberculosis* urease, as is the case with other ureases (37). Phenylphosphorodiamidate was also a potent inhibitor of *M. tuberculosis* urease, although it showed a somewhat higher 50% inhibitory concentration than we observed for jack bean or *B. pasteurii* urease (80 versus 8 nM).

(v) Stability of *M. tuberculosis* urease. *M. tuberculosis* urease is remarkably stable against heat treatment. No loss of enzyme activity occurred with 30 min of incubation at temperatures between 22 and 60°C. However, *M. tuberculosis* urease was progressively inactivated at temperatures above 60°C. Consistent with these results, *M. tuberculosis* urease showed steadily increasing reaction velocities as the reaction temperature was increased from 0 to 60°C and, at higher temperatures, reaction velocities rapidly decreased as the enzyme was inactivated (data not shown). We have found that jack bean and *B. pasteurii* ureases are less stable against heating than is *M. tuberculosis* urease and lose activity at temperatures above 45°C (data not shown). Although *M. tuberculosis* urease may be exposed to temperatures as high as 42°C in a human host with fever, it is unlikely that the urease would be exposed to temperatures higher than this in its natural environments.

a

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M. tuberculosis    MRLTPHEQERLLLSYAAELARRRRARGLRLNHPEAIAVIADHILEGARDGRTVAE   55
Bacillus sp.       MKLTSREMEKLMIVVAADLARRRKERGLKLNYPEAVAMITYEVLEGARDGKTVAQ   55
K. aerogenes       MELTPREKDKLLLFTAALVAERRLARGLKLNYPESVALISAFIMEGARDGKSVAS   55
P. mirabilis       MELTPREKDKLLLFTAGLVAERRLAKGLKLNYPERVALISCAIMEGAREGKTVAQ   55

M. tuberculosis    LMASGREVLGRDDVMEGVPEMLAEVQVEATFPDGTKLVTVHQPIA            100
Bacillus sp.       LMQYGA-TLTKEDVMEGVAEMIPDIQIEATFPDGTKLVTVHDPIR             99
K. aerogenes       LMEEGR-HLTREQVMEGVPEMIPDIQVEATFPDGSKLVTVHNPII             99
P. mirabilis       LMSEGR-TLTAEQVMEGVPEMIKDVQVECTFPDGTKLVSIHSPIV             99
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b

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M. tuberculosis    MIPGEIFYGSGDIEMNAAALSRLQMR---IINAGDRPVQVGSHVHLPQANRALSFD   53
Bacillus sp.       MIPGEYVLKKEPILCNQNK----QTIKIRVLNRGDRPVQVGSHFHFFEVNQSLQFH   52
K. aerogenes       MIPGEYHVKPGQIALNTGR----ATCRVVVENHGDRPIQVGSHYHFAEVNPALKFD   52
P. mirabilis       MIPGEIRVNAALGDIELNAGRETKTIQ--VANHGDRPVQVGSHYHFYEVNEALRFA   54

M. tuberculosis    RATAHGYRLDIPAATAVRFEPGIPQIV-GLVPLGGRREVPGLTLNPPGRLDR      105
Bacillus sp.       REKAFGMRL-NPAGTAVRFEPGDAKEV-EIIPFSGERKVYGLNNVTNGSVEMGKRK  106
K. aerogenes       RQQAAGYRL-NPAGTAVRFEPGQKREV-ELVAFAGHRAVFGFRGEVMGPLEVNDE   105
P. mirabilis       RKETLGFRN-IPAGMAVRFEPGQSRTVDELVAFAGKREIYGFHGKVMGKLESEKK   108
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FIG. 6. Protein sequence alignments of *M. tuberculosis* urease subunits A (a) and B (b) with those of other bacteria. Conserved residues are indicated in boldface type. Dashes indicate gaps inserted in the sequences to improve the alignments.

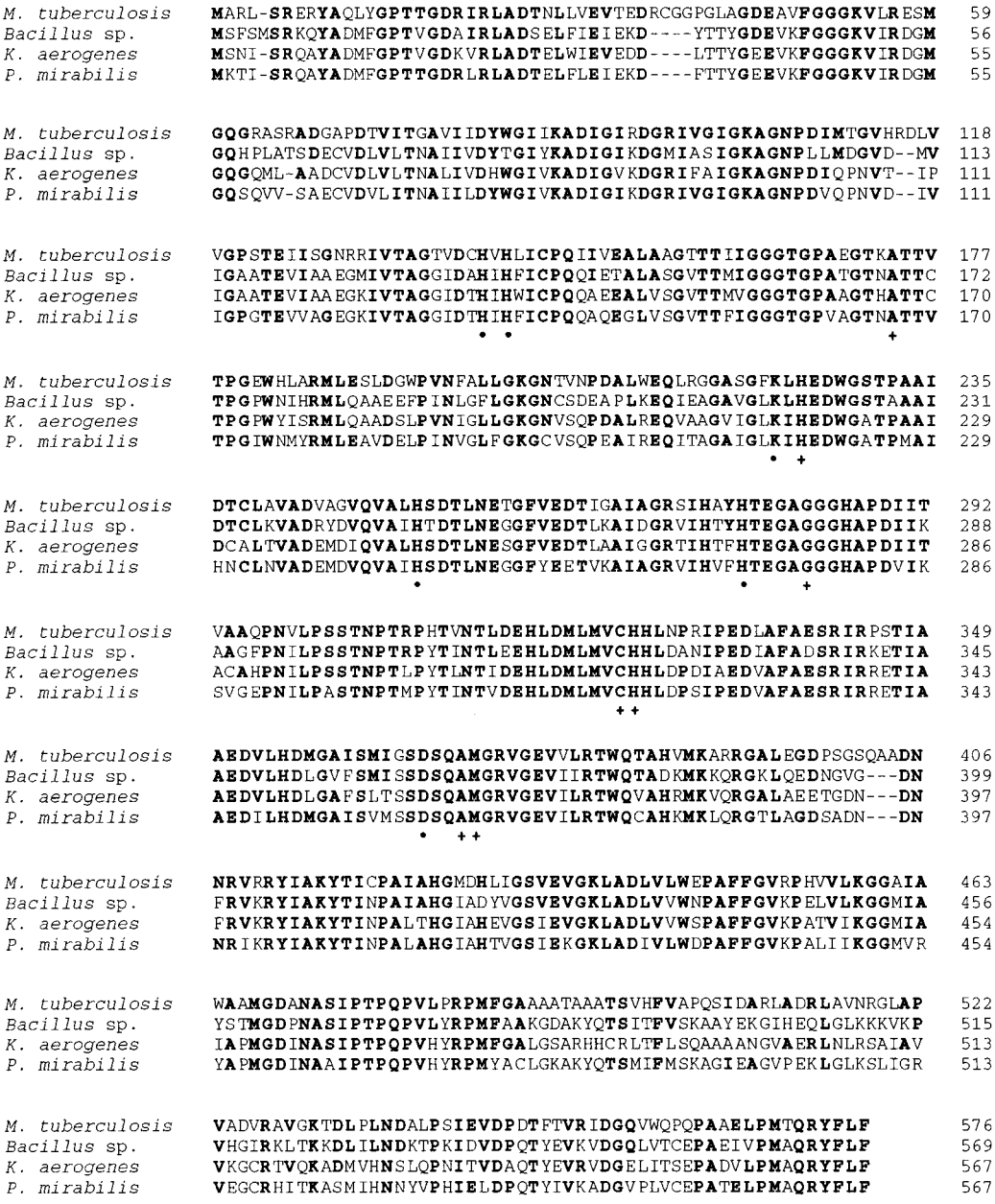

FIG. 7. Protein sequence alignments of *M. tuberculosis* urease C subunit with those of other bacteria. Conserved residues are indicated in boldface type. Dashes indicate gaps inserted in the sequences to improve the alignments. Conserved residues implicated in coordinating the two Ni^{2+} ions at the active site (His-141, -143, -252, and -278; Asp-366; and Lys-223) are indicated with a dot, and conserved residues lining the active site (Ala-174, His-225, Gly-283, Cys-325, His-326, Ala-369, and Met-370) are indicated with a plus sign.

Treatment of *M. tuberculosis* urease with 4 M urea caused no loss of enzyme activity. In addition, the gel filtration chromatographic elution profile of urease on Sephacryl-S300 HR was identical in the presence or absence of 4 M urea (data not shown).

Induction of *M. tuberculosis* urease by exposure to low-nitrogen conditions. Urease activity of *M. tuberculosis* is easily measured even in nitrogen-rich media, such as 7H9 medium (which contains nitrogen in the form of 3.8 mM ammonium sulfate and 3.4 mM glutamic acid) or Sauton's medium (which contains 15 mM asparagine). However, expression of *M. tuberculosis* urease activity was induced almost 10-fold in modified Sauton's medium with a decreased asparagine concentration (Table 2). The increased expression of *M. tuberculosis* urease in response to nitrogen starvation is consistent with a role for the enzyme in nitrogen acquisition.

Cloning and sequencing of the *M. tuberculosis* urease gene cluster. We constructed a genomic library by partial digestion of *M. tuberculosis* chromosomal DNA with *Bam*HI and *Hin*dIII, ligation of the fragments into pUC19, and transformation of *E. coli* DH5α. A set of degenerate 45-mer oligodeoxynucleotides based on the N-terminal protein sequence (YAQLYG PTTGDRIRL, residues 8 to 22) of the *M. tuberculosis* urease C subunit was synthesized and used to screen the genomic li-

a

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M. tuberculosis   MTSLAVLLTLADSRLPTGAHVHSGGIEEAIAAGMVTGLATLEAFLKRRVRTHGLLTAS   58
Bacillus sp.      MNRLLSLFQLCDSNFPSGSFSHSFGLETYIQEKVITDKESFKNAISVYIRKQLFFTEG   58
K. aerogenes      MSTAEQRLRLMQLASSNLPVGGYSWSQGLEWAVEAGWVLDVAAFERWQRRQMTEGFFT   58
P. mirabilis      MMLADLRL--YQLVSPSLPVGAFTYSQGLEWAIEKGWVCSAETLSDWLSAQMTGTLAT   56

M. tuberculosis   IAAAVHRGELAVDD---------------ADRETDARTPAPAARHASRSQGRGLIRLARR   103
Bacillus sp.      LACILAYEAMEKNEPSALVELDHILFASNVAQETRSGNQRMGERMAKLCVDLYPSPILIE   118
K. aerogenes      VDLPLFARLYRACEQGDIAAAQRWTAYLLACRETRELREEERNRGAAFARLLSDWQPDCP   118
P. mirabilis      LELPILRQLQTSLAKGDSDTVKYWCDFMVASRETKELRQEERQPGIAFPRLLPQLGIELD   116

M. tuberculosis   VWPDSGWEELGPRPHLAVVAGRVGALSGLAPEHNALHLVYITMTGSAIAAQRLLALDPA   162
Bacillus sp.      YTNRI--KEKKAYGHSAIVFAIVAYHLKVTKE-TAV-GAYLFANVSALVQNAVRGI-PI   172
K. aerogenes      PPWRSLCQQSQLAGMAWLGVRWRIALPEMALSLGYSWI--ESAVM-AGVKLVPFGQQAA   174
P. mirabilis      DTLQQRVKQTQLMAFALAAVHWHIDS-EK-LCCAYVWGWLENTVMS-GVKLVPLGQSAG   172

M. tuberculosis   EVTVVTFQLSELCEQIAQEATAGLADLSDPLLDT------LAQ-RHDERVRPLFVS     211
Bacillus sp.      GQTDGQRILVEIQPLL-EEGVRTISQLPKEDLGAVSPGMEIAQMRHERLNVRLFMS     227
K. aerogenes      QQLILRLCDHYAAEMPRALAAPDGDIGSATPLAA------IASARHETQYSRLFRS     224
P. mirabilis      QKMLFALAEQIPAIVELSAHWPQEDIGSLRQLK                             205
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b

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M. tuberculosis   MATHSHPHSHTVPARPRRVRKPGEPLRIGVGGPVGSGKTALVAALCRQLRGELSLAV   57
Bacillus sp.      M----------------------EPIRIGIGGPVGAGKTMLVEKLTRAMHKELSIAV   35
K. aerogenes      MNSYKH-----------------PLRVGVGGPVGSGKTALLEALCKAMRDTWQLAV   39
P. mirabilis      MQEYNQ-----------------PLRIGVGGPVGSGKTALLEVLCKAMRDSYQIAV   39

M. tuberculosis   LTNDIYTTEDADFLRTHAVLPDDRIAAVQTGGCPHTAIRDDITANLDAIDELMAAH   113
Bacillus sp.      VTNDIYTKEDAQFLLKHGVLPADRVIGVETGGCPHTAIREDASMNFPAIDELKERH   91
K. aerogenes      VTNDIYTKEDQRILTEAGALAPERIVGVETGGCPHTAIREDASMNLAAVEALSEKF   95
P. mirabilis      VTNDIYTQEDAKILTRAQALDADRIIGVETGGCPHTAIREDASMNLAAVEELAMRH   95

M. tuberculosis   DALDLILVESGGDNLTATFSSGLVDAQIFVIDVAGGDKVPRKGGPGVTYSDLLVV   168
Bacillus sp.      PDLELIFIESGGDNLAATFSPELVDFSIYIIDVAQGEKIPRKGGQGMIKSVLFII   146
K. aerogenes      GNLDLIFVESGGDNLSATFSPELADLTIYVIDVAEGEKIPRKGGPGITKSDFLVI   150
P. mirabilis      KNLDIVFVESGGDNLSATFSPELADLLFMLIDVAEGEKIPRKGGPGITHPDMMVI   150

M. tuberculosis   NKTDLAALVGADLAVMARDADAVRDGRPTVLQSLTEDPAASDVVAWVRSQLAADGV   224
Bacillus sp.      NKIDLAPYVGASLEVMERDTLAARGDKPYIFTNLKDEIGLAEVLEWIKTNALLYGLES 204
K. aerogenes      NKTDLAPYVGASLEVMASDTQRMRGDRPWTFTNLKQGDGLSTIIAFLEDKGMLGK    205
P. mirabilis      NKIDLAPYVGASLEVMEADTAKMRPVKPYVFTNLKEKVGLETIIDFIIDKGMLRR    205
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FIG. 8. Protein sequence alignment of *M. tuberculosis* urease accessory molecules F (a) and G (b) with those of other bacteria. Conserved residues are indicated in boldface type. Dashes indicate gaps inserted in the sequence to improve the alignments. The ATP- and GTP-binding motif (P-loop, GXGGPVGXGKT) of the urease G accessory molecule is conserved (underlined).

brary. Restriction analysis of the positive clones selected from colony hybridization indicated that all the positive clones contained a 4.5-kb *Bam*HI insert. DNA sequencing of one of the positive clones, pTBU5, revealed the presence of complete *ureA* and *ureB* genes and two-thirds of the 5′ region of the *ureC* gene. To isolate DNA fragments containing the complete urease gene cluster, we constructed a cosmid library by partial digestion of *M. tuberculosis* genomic DNA with *Eco*RI, ligation into *Eco*RI-digested pHC79 vector, and transformation of *E. coli* DH5α. The cosmid library was screened with the 4.5-kb DNA fragment as a probe. In all positive clones examined, an 8.5-kb *Eco*RI fragment hybridized with the 4.5-kb DNA probe, indicating the presence of the *M. tuberculosis* urease gene cluster on this 8.5-kb *Eco*RI insert.

Sequence analysis of the 8.5-kb *Eco*RI fragment of *M. tuberculosis* cloned into pHC79 revealed three genes corresponding to the urease A, B, and C subunits and two genes for the urease accessory molecules F and G (11, 20, 27) (Fig. 4 to 8). The guanine and cytosine contents of these five genes ranged from 65 to 71%, which is typical for mycobacteria. The five genes of the *M. tuberculosis* urease gene cluster span 3.7 kb on the chromosome, are closely linked, and are organized in a fashion similar to those of urease gene clusters of other bacteria (Fig. 4). *ureA* gene shares one nucleotide of the codon for its last amino acid residue (alanine) with the codon for the first amino acid residue (methionine) of the *ureB* gene (Fig. 5). The first codon of both the *ureC* and *ureF* genes shares one nucleotide with the stop codon of the preceding gene (*ureB* and *ureC* genes, respectively). The first codon of the *ureG* gene, the last gene identified in the *M. tuberculosis* urease gene cluster, begins only 10 nucleotides downstream of the stop codon of the *ureF* gene (Fig. 5).

Sequence analysis of the 8.5-kb *Eco*RI fragment containing the *M. tuberculosis* urease gene cluster revealed no genes corresponding to the genes for the D or E accessory molecules of other bacteria. In the cases of *K. aerogenes* (16) and *P. mirabilis* (11), the *ureD* gene is located immediately upstream of the *ureA* gene. Instead, in the case of *M. tuberculosis*, an open

reading frame with 34% identity to the thioesterase of an *Arthrobacter* sp. (34) was located upstream of the *ureA* gene. We have identified three open reading frames downstream of the *ureG* gene. However, they have no significant similarity to other genes in GenBank.

Sequence comparison and homology. The *M. tuberculosis ureA* gene codes for a 100-amino-acid polypeptide (Fig. 6) with a predicted molecular mass of 11.1 kDa and a pI of 6.2. Its sequence is 62, 61, and 60% identical to the sequences of the urease A subunit of *B. pasteurii*, *K. aerogenes*, and *P. mirabilis*, respectively (11, 20, 27), and 56% identical to the sequence of the first 102 residues of the small subunit of *H. pylori* urease (13). The *M. tuberculosis ureB* gene codes for a 104-amino-acid polypeptide (Fig. 6) with a predicted molecular mass of 11.2 kDa and a pI of 10.9. It is 41 to 45% identical to the subunit B sequences in other bacteria and 41% identical to the corresponding residues 107 to 214 of the small subunit of *H. pylori*. The most highly conserved region of the urease B subunit, located in the middle of the sequence from residues 32 to 78, shows 58 to 60% identity to the corresponding sequences in other bacteria. The *M. tuberculosis ureC* gene codes for a 576-amino-acid polypeptide (Fig. 7) with a predicted molecular mass of 60.8 kDa. It is 58 to 60% identical to the sequence of the urease C subunit of *B. pasteurii*, *K. aerogenes*, and *P. mirabilis* (11, 20, 27) and 53% identical to the sequence of the large subunit of *H. pylori* urease (13). X-ray crystallographic analysis of *K. aerogenes* urease (10) has identified four histidine residues, an aspartate, and a carbamylated lysine as important in coordinating the two nickel ions at the active site. These residues are conserved in the C subunit of *M. tuberculosis* urease (His-141, -143, -252, and -278; Asp-366; and Lys-223) as well as in other ureases. Additional residues of the *K. aerogenes* urease C subunit identified as lining the active site and proposed to be important in substrate binding and catalysis (10) are also conserved in *M. tuberculosis* urease (Ala-174, His-225, Gly-283, Cys-325, His-326, Ala-369, Met-370) as well as in other ureases.

The *M. tuberculosis* urease accessory molecule F (Fig. 8) has a predicted molecular mass of 22.3 kDa and exhibits limited homology to the amino acid sequence of accessory molecule F in other bacteria. The open reading frame encoding the *M. tuberculosis* accessory molecule G predicts a protein of 224 amino acids (Fig. 8) with a molecular mass of 22 kDa and an amino acid sequence 46 to 48% identical to the sequences of accessory molecule G from other bacteria. An ATP- and GTP-binding motif (P-loop; GXGGPVGXGKT) is present within the accessory molecule G sequence of *M. tuberculosis* as well as the corresponding sequences of other bacteria (11, 32). Compared with the corresponding sequence of *B. pasteurii*, the sequence of *M. tuberculosis* accessory molecule G has an additional 22 amino acids at its amino terminus, in which a histidine-rich sequence can be found (THSHPHSH).

DISCUSSION

Urease is present in many species of mycobacteria, and its presence or absence is routinely used in the speciation of mycobacteria (19). Although urease has been purified from a number of bacterial sources and characterized and its genes have been cloned and sequenced (26), this work represents the first purification, characterization, and genetic analysis of urease of *M. tuberculosis* or of any mycobacterial urease. We purified the enzyme 1,000-fold by a combination of anion-exchange chromatography, gel filtration chromatography, and hydrophobic-interaction chromatography. Of these three steps, hydrophobic-interaction chromatography was consistently the most powerful method in separating *M. tuberculosis* urease from other proteins present in the cellular extracts.

Urease has been implicated as a virulence factor for several other pathogenic microorganisms. First, ammonia production by urease of urinary pathogens, such as *P. mirabilis*, has been implicated in pathogenesis by virtue of its toxicity to renal epithelium, promotion of urinary stone formation, and participation in complement inactivation (2, 7). Second, urease of *H. pylori* has been implicated in pathogenesis by virtue of its role in alkalinizing the bacterium's microenvironment in the stomach and its toxicity to stomach epithelium (35). In the case of *M. tuberculosis*, urea is likely readily available to the bacterium in both its intracellular and extracellular locations within the host. Ammonia generated by the action of urease may be of considerable importance in alkalinizing the microenvironment of the organism and in preventing phagosome-lysosome fusion and phagosome acidification. In addition, ammonia generated by the action of urease should be available to the organism for assimilation of nitrogen into biomolecules. The concerted action of urease and glutamine synthetase (9), which are both induced by conditions of low nitrogen, should serve to scavenge and assimilate environmental nitrogen. The availability of nitrogen in the *M. tuberculosis* phagosome is unknown. However, the phagosome, in which *M. tuberculosis* multiplies, is remarkable for its low fusogenicity. Markers of fluid-phase endocytosis added to infected cells either before or after infection of the cell with *M. tuberculosis* do not enter the *M. tuberculosis* phagosome (1, 4, 41). Therefore, nitrogen nutrients may be limiting to the *M. tuberculosis* organism inside the relatively nonfusogenic phagosome, and urease may play an important role in nitrogen acquisition by *M. tuberculosis* at this site.

Urease is present in most pathogenic mycobacteria, such as *M. tuberculosis* and *Mycobacterium bovis*. Urease is absent from some nonpathogenic mycobacteria, such as *Mycobacterium xenopi*, *Mycobacterium gordonae*, and *Mycobacterium triviale*, but is present in others, such as *Mycobacterium smegmatis*, *Mycobacterium vaccae*, and *Mycobacterium phlei* (19). In the case of the nonpathogenic species *M. smegmatis*, we have found that the level of urease activity in the crude bacterial extract is 11-fold greater than it is in *M. tuberculosis*. In addition, we have found that the K_m of urease from *M. smegmatis* is 0.3 mM, which is equal to the K_m of *M. tuberculosis* urease (4a). In view of these findings, it is unlikely that urease expression alone is sufficient to explain the greater virulence of *M. tuberculosis*. Nevertheless, urease expression may be an important contributor to virulence mechanisms, for example, inhibition of phagosome-lysosome fusion and phagosome acidification.

Assembly of active urease is a complex process and involves several urease accessory molecules. Deletions or mutations in genes coding for the urease accessory molecules have been found to abolish urease activity (16, 20). Sequence analysis of the *M. tuberculosis* urease gene cluster has revealed genes for the urease accessory molecules F and G but not for urease accessory molecule D or E. In other bacteria, urease accessory molecule D appears to act as a chaperone for assembly of the urease molecule (30), and urease accessory molecule E appears to function as a nickel donor to the urease apoprotein (17). In all other bacteria for which the urease gene cluster has been studied, the *ureD* and *ureG* genes have been found closely linked within the urease gene cluster. However, we cannot rule out the possibility that one or more of the three open reading frames downstream of the *ureG* gene codes for accessory molecules with functions corresponding to those of accessory molecules D and E of other bacteria, despite the absence of protein sequence homology, or that the functions provided by D

and E are encoded by genes not linked to the urease cluster. As a precedent for this, in the case of *H. pylori*, a gene for a high-affinity Ni^{2+} transport protein (*nixA*, which is homologous to the *ureH* gene of *B. pasteurii*) has been found outside of the urease gene cluster (24).

Given its likely importance to pathogenesis, urease is a potential target for chemotherapeutic intervention in tuberculosis. More knowledge of the structure and function of urease may allow the development of rational strategies to interfere with its pathogenetic activities.

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